‘*Candidatus* Phytoplasma palmicola’, a novel taxon associated with a lethal yellowing-type disease (LYD) of coconut (*Cocos nucifera* L.) in Mozambique

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In this study, the taxonomic position and group classification of the phytoplasma associated with a lethal yellowing-type disease (LYD) of coconut (*Cocos nucifera* L.) in Mozambique were addressed. Pairwise sequence similarity values based on alignment of near full-length 16SrRNA genes (1530 bp) revealed that the Mozambique coconut phytoplasma (LYDM) shared 100% identity with a comparable sequence derived from a phytoplasma strain (LDN), responsible for Awka wilt disease of coconut in Nigeria, and shared 99-99.6% identity with 16S rRNA sequences from strains associated with Cape St. Paul wilt (CSPW) disease of coconut in Ghana and Côte d'Ivoire. Similarity scores further determined the 16S rRNA gene of LYDM phytoplasma to share <97.5% sequence identity with all prior descriptions of '*Ca*. Phytoplasma' species. Presence of unique regions in the 16S rRNA distinguished LYDM phytoplasma from all currently described '*Candidatus* Phytoplasma' species, justifying its recognition as reference strain of a novel taxon, '*Candidatus* Phytoplasma palmicola'. Restriction fragment length polymorphism (RFLP) profiles of the F2n/R2 portion (1251 bp) of the 16S rRNA gene and pattern similarity coefficient values delineated coconut LYDM phytoplasma strains from Mozambique as new members of established group 16SrXXII, subgroup A (16SrXXII-A). Similarity coefficients of 0.97 were obtained for comparisons between subgroup 16SrXXII-A strains and CSPW phytoplasmas from Ghana and Côte d'Ivoire. On this basis, CSPW phytoplasma strains were designated as members of a new subgroup, 16SrXXII-B.

Coconut (*Cocos nucifera* L.) is a member of the palm family (*Arecaceae*) and sole recognized species in the genus *Cocos* (Persley, 1992). The general consensus has been that coconut originated in the Indo-Pacific region either around Melanesia, Malesia or land masses bordering the Indian Ocean, and has recorded histories spanning 3 millennia in Asia Minor, at least two millennia in East Africa, and about five centuries in West Africa and the Americas (Clement *et al*., 2013; Ohler, 1984; Schuiling and Harries, 1994). Pan-tropical in present day distribution, this species is often cultivated in environments where few alternative crops can thrive, providing a sustainable source of food and shelter, as well as an important source of rural income to small farmers (Persley, 1992). Unfortunately, lethal diseases caused by phytoplasmas pose serious threats to sustainability of coconut cultivation worldwide.

Phytoplasmas are unculturable, pleomorphic bacteria that belong to the class Mollicutes (Bertaccini, 2007; Gasparich, 2010; Hogenhout *et al*., 2008). They are the presumed aetiological agents of yellows-type diseases affecting at least 1000 plant species worldwide (McCoy *et al*., 1989; Lee *et al*., 2000; Seemüller *et al*., 1998). In nature, these bacteria alternate passage between plant and insect hosts in which they propagate and persist and depend upon plant to plant transmission by phloem-feeding insect vectors of the order Hemiptera, primarily, leafhoppers, planthoppers and psyllids (D'Arcy & Nault, 1982; Weintraub & Beanland, 2006). Plant diseases associated with phytoplasma infection are often characterized by symptoms indicative of disturbances in the balance of plant growth regulators (Aguilar *et al*., 2009; Martinez *et al*., 2000). From various molecular studies, it is well established that phytoplasmas possess small A+T rich genomes (Kollar & Seemüller, 1989; Tran-Nguyen & Gibb, 2007) ranging from 530-1,350 kb in size (Neimark & Kirkpatrick, 1993; Marcone *et al*., 1999), and a gene complement indicative of only a limited number of metabolic pathways (Andersen *et al*., 2013; Bai *et al*., 2006; Kube *et al*., 2008; Oshima *et al*., 2004; Tran Nguyen *et al*., 2008). These features may be attributable in part to phage-associated genome reductions during an ongoing evolutionary adaptation of phytoplasmas to their plant and insect hosts (Davis *et al*., 2005; Jomantiene *et al*., 2007; Wei *et al*., 2008).

Based on their identification and classification by restriction fragment length polymorphism (RFLP) analysis of 16S ribosomal RNA gene sequences amplified by polymerase chain reaction (PCR) assay (Lee *et al*., 1998; 2000), phytoplasmas constitute a genetically diverse taxon of trans-kingdom parasites consisting of at least 28 groups and numerous subgroups (Wei *et al*., 2007). Furthermore, groups defined by RFLP typing are largely supported by phylogenetic analysis of 16S rDNA sequences upon which the taxonomy of phytoplasmas is currently based (IRPCM, 2004). Thirty-six '*Candidatus* Phytoplasma' species descriptions have thus far been formally described (Davis *et al*., 2013; Nejat *et al*., 2013; Quaglino *et al*., 2013), using '*Candidatus'* concepts for naming incompletely described microorganisms (IRPCM, 2004; Murray & Stackebrandt, 1995).

In Africa, reliable reports of coconut diseases, later attributed to phytoplasmas (Dabek *et al*., 1976; Dollet *et al*., 1977; Dollet & Giannotti, 1976; Nienhaus & Steiner; 1976), date back to the

early 1900s (Eden-Green, 1997). In West Africa, these diseases are known by various local or regional names. They include Awka wilt disease in Nigeria (Ekpo and Ojomo, 1990; Osagie & Asemota, 1997), Cape St. Paul wilt (CSPW) disease in Ghana (Ofori & Nkansah-Poku, 1997; Danyo, 2011; Sangaré *et al*., 1992) and Côte d'Ivoire (Konan Konan *et al*., 2013), Kaïncopé in Togo (Meiffren, 1951; Agounké, 1997) and kribi disease in Cameroun (Dollet *et al*., 1977). In East Africa, a lethal disease of coconut is present along the coastal belt of Tanzania and in Kenya (Schuiling & Mpunami, 1992a), while a similar disease is active in Mozambique (Bonnot *et al*., 2010). Collectively, these diseases all share a succession of symptoms closely resembling lethal yellowing, a name first used by Nutman & Roberts (1955) to denote a fatal phytoplasma-associated disease of coconut in Jamaica (Beakbane, *et al*., 1972; Heinze *et al*., 1972; Plavsic-Banjac *et al*., 1972) that has since spread to other parts of the Caribbean basin (Ntushelo *et al*., 2013). Seemingly identical aetiologies and shared symptoms initially supported the view of a common origin for these coconut lethal yellowing-type diseases (LYD) (Ogle & Harries, 2005; Ollagnier & Weststeijn, 1962). However, differences in epidemiology (Danyo, 2011; Nkansah-Poku *et al*., 2009; Philippe *et al*., 2009; Schuiling *et al*., 1992b) and coconut ecotype susceptibility (Dery *et al*., 2008; Schuiling *et al*., 1992c) indicated that coconut-associated phytoplasmas in Africa were probably distinct from those affecting palms in the Caribbean basin (Eden-Green, 1997; Eziashi & Omamar, 2010).

The involvement of mutually distinct phytoplasmas with coconut diseases in West Africa (Nigeria and Ghana) versus East Africa (Kenya and Tanzania) was clearly demonstrated by specific amplification of 16S rRNA genes in PCR assays employing primer pairs based upon unique sequences in rRNA operons of the respective pathogens and augmented by RFLP analysis of PCR products (Tymon *et al*., 1997; 1998; Wei *et al*., 2007). In addition, PCRs incorporating primer pair G813/AKSR, designed to amplify DNA of West African coconut phytoplasmas (Tymon *et al*., 1997), were also later found to amplify rDNA sequences from coconuts with LYD symptoms in Mozambique, East Africa (Mpunami *et al*., 1999). However, not all diseased palms in Mozambique yielded phytoplasma positive results when PCRs were primed by G813/AKSR, an outcome that upon further study was attributed to variability in the region of the 16-23S intergenic spacer region (ISR) sequence corresponding to primer AKSR (Dollet *et al*., 2009). Phylogenetic analysis of near full-length 16S rRNA gene sequences

revealed that coconut phytoplasmas from West Africa (Nigeria and Ghana) and East Africa (Tanzania) formed two distinct subclades designated as (xii) and (xiv), respectively (Tymon *et al.*, 1998); both subclades were separate from subclade (vii), which encompassed coconut phytoplasmas from the Caribbean region (Gundersen *et al*., 1994). Based on these findings, it was informally proposed that these subclades represented three separate '*Candidatus* Phytoplasma' species (IRPCM, 2004). Further support for this proposal has since been obtained from phylogenetic analysis of rrn operon sequences consisting of the 16-23S ISR and 5'-end of the 23S rRNA gene, and by similar analysis of *sec*A gene sequences, both of which also differentiated African and Caribbean coconut phytoplasmas into three distinct clusters or groups (Hodgetts *et al*., 2008), but formal descriptions of these taxa remain to be published.

Coconut palms with symptoms indicative of LYD were reported near Quelimane in Zambesia Province, Mozambique during 2007 (Dollet *et al*., 2009) and on 2013 were observed for the first time, in the Grand Lahou district on the southern coastal belt of Côte d'Ivoire (Konan Konan *et al*., 2013). Although the coconut-infecting phytoplasmas detected in both countries were judged most similar to strains previously associated with Awka wilt disease in Nigeria and CSPW disease in Ghana (Dollet *et al*., 2011; Konan Konan *et al*., 2013), their precise taxonomic positions and group affiliations were not determined. In this communication, we report results from nucleotide and phylogenetic analysis of 16S rRNA gene sequences, and propose that phytoplasma strains associated with coconut LYD in Mozambique, Nigeria, Ghana and Côte d'Ivoire, represent a distinct species level lineage and novel taxon, '*Candidatus* Phytoplasma palmicola'.

Mozambique coconut phytoplasma (LYDM) represents a novel taxon

To characterize phytoplasmas associated with coconut LYD in Mozambique and Côte d'Ivoire, DNA fragments consisting of a portion of the *rrn* operon (1.8 kb) were amplified by PCR assay employing phytoplasma universal primer pair P1 (Deng & Hiruki, 1991) and P7 (Smart *et al*., 1996) and CTAB extractions of total DNAs (Harrison *et al*., 2013) from inflorescence or stem tissues as template. Resulting PCR products, each comprising a near full-length 16S rRNA gene, 16-23S rRNA intergenic spacer and partial 23S rRNA gene, were purified separately on spin columns using a Wizard PCR Preps purification system (Promega, Madison,WI) and sequenced

directly on automated equipment at the University of Florida's Core Genomics Service laboratory to obtain at least 3X coverage per base position. Sequences obtained from three diseased palms in both Mozambique and Côte d'Ivoire were archived in the GenBank nucleotide database as accessions EU549768, KF751386, KF751388 and KF364359, KF387570, KF419286, respectively. The portion of the *rrn* operon corresponding to the 16S rRNA gene (positions 1..1530) in each accession contains a sequence $_{266}$5'-CAAGACGATGATGTGTAGCTGGACT-3'$_{290}$ matching the signature sequence (5'-CAAGAYBATKATGTKTAGCYGGDCT-3') that defines the taxa in the provisional genus '*Candidatus* Phytoplasma' (IRPCM, 2004).

To determine their taxonomic position, near full-length (1530 bp) 16S rRNA gene sequences derived from Mozambique and Côte d'Ivoire phytoplasma strains, from other West African coconut phytoplasmas, from reference strains of all formally described '*Ca* Phytoplasma' species, and from strains representative of additional phytoplasma groups, were retrieved from the GenBank nucleotide database. A global alignment of sequences was constructed using CLUSTAL W in the MEGALIGN program (DNASTAR, Madison, WI) and queried using the sequence distances option to obtain % identity values. The results confirmed that all three LYDM phytoplasma sequences from Mozambique were mutually identical as were CSPW phytoplasma sequences obtained from Côte d'Ivoire in this study. In addition, LYDM phytoplasma sequences shared 100% identity with that of Awka wilt phytoplasma strain LDN from Nigeria (GenBank accession Y14175), for which a provisional name of '*Candidatus* Phytoplasma cocosnigeriae' was previously proposed (IRPCM, 2004). By comparison, LYDM sequences shared 99.6% identity with those of CSPW-associated phytoplasmas from Ghana (accessions Y13912, JQ868442) and Côte d'Ivoire (accessions KC999037, KF364359, KF387570, KF419286), but less than 97.5% identity with all previously described '*Ca*. Phytoplasma' species reference strains.

Differences in sequence identity (%) values between near full length 16S rRNA gene sequences (1530 bp) of LYDM phytoplasmas and those of Côte d'Ivoire strains were attributable to a total of six single nucleotide polymorphisms (SNPs) in base positions as follows: A/G$_{252}$, T/C$_{262}$, T/C$_{619}$, T/C$_{983}$, T/G$_{1006}$ and A/G$_{1019}$ in this study. These same six SNPs were identified when

LYDM sequences were aligned with a comparable length (1530 bp) 16S rRNA gene sequence of CSWP strain LDG (Y13912) and with lesser length 16S rRNA gene sequences of strains CSPWB (JQ868442) from Ghana and CILY (KC999037) from Côte d'Ivoire that are 1508 bp and 1251 bp in length, respectively. Strain LDG (Y13912) also possessed a putative SNP, namely G/T_{646}, that was absent from Mozambique strains and from all other West African coconut phytoplasmas. The putative SNP G/T_3 was unique to the CSPWB (JQ868442) sequence while the following seven SNPs with their respective positions, G/A_6, A/G_7, C/T_8, A/G_{16}, C/T_{49}, A/G_{295} A/T_{1226} within the sequence derived from strain CILY (KC999037), were absent from all other West African coconut strains and from the Mozambique strains.

The '*Ca* Phytoplasma' species most closely related to LYDM phytoplasmas was '*Candidatus* Phytoplasma castaneae' whose 16S rRNA gene sequence shared 94% identity with the former LYDM query sequences. Guidelines for the recognition of a novel '*Ca*. Phytoplasma' species (IRPCM Phytoplasma/Spiroplasma Working Team – Phytoplasma Taxonomy Group, 2004) state that a strain can be described as a novel '*Ca*. Phytoplasma' species if its 16S rRNA gene sequence has <97.5% similarity to that of any previously described '*Ca*. Phytoplasma species (IRPCM, 2004). Our data illustrate that LYDM phytoplasmas meet the criterion for recognition as a novel '*Ca*. Phytoplasma' species for which we propose the name '*Candidatus* Phytoplasma palmicola'.

The global alignment of 16S rRNA gene sequences was assessed further to identify signature sequences unique to LYDM phytoplasmas. Comparisons of the aligned sequences revealed at least four unique regions. They include: $_{169}$5'-AATGAGGCATCTCGTTAT-3'$_{186}$, $_{196}$5'-ACCTTCGCAAGAAGGT-3'$_{211}$, $_{448}$5'-CGCTAGTGGAAAAACTAGT-3'$_{466}$ and $_{1237}$5'-CAGCTGAAACGTGAGTTGTTAGCT-3'$_{1260}$ which differ from three to nine, from four to nine, from five to nine and from three to 10 base positions, respectively, from the corresponding regions in the 16S rRNA genes of all previously described '*Ca*. Phytoplasma' species.

New phytoplasma subgroup 16SrXXII-B

To augment description of LYDM phytoplasmas and to further clarify their interrelationships with strains affecting coconut palms in West Africa, the F2n/R2 portion of the 16S rRNA gene

(Lee *et al*., 1998) was subjected to virtual restriction site analysis employing 17 recommended restriction endonucleases (Zhao *et al*., 2009b). Resulting RFLP patterns were visualized and compared on virtual 3% gels using pDRAW32 (AcaClone software, http://www.acaclone.com). Differences in fragment profiles between strains were evident for digestions with *Hae*III only. This endonuclease cuts the F2n/R2 sequence of LYDM and Awka wilt (LDN) phytoplasmas at positions 121 and 187, producing fragments of 66, 121, and 1064 bp in size, respectively. By comparison, the endonuclease cuts F2n/R2 sequences of all Ghana and Côte d'Ivoire strains just once at position 187 producing fragments of 187 and 1064 bp, respectively. The base difference at SNP T/C_{262} eliminates a restriction site for the latter strains that all three LYDM strains and Awka wilt phytoplasmas possess. The F2n/R2sequences were also queried using the *i*PhyClassifier program (Zhao *et al*., 2009b). A similarity coefficient value of 1.0 was obtained in all pairwise comparison of LYDM strains with Awka wilt strain LDN from Nigeria. The latter strain was previously identified and classified as sole member of RFLP group 16SrXXII, subgroup A (i.e. 16SrXXII-A) (Wei *et al*., 2007). These values affirmed LYDM phytoplasmas as new members of this established subgroup. However, virtual RFLP analysis of sequences derived from strains LDG (Y14175) and CSPWB (JQ868442) from Ghana, as well as CILY (KF387570) and additional Côte d'Ivoire strains (KF364359, KF387570, KF419286) from this study, each generated a similarity coefficient value of 0.97 in all pairwise comparisons with LYDM or LDN phytoplasmas. Thus, coconut phytoplasmas resident in Ghana and Côte d'Ivoire were judged most similar to those of subgroup 16SrXXII-A; a value of 0.97 indicates that the latter strains warrant classification as a new subgroup within group 16SrXXII. Therefore, we assign LDG, CSPB and CILY and additional Côte d'Ivoire strains to subgroup 16SrXXII-B and members of this subgroup as '*Ca*. Phytoplasma palmicola'-related strains.

Description of '*Candidatus* Phytoplasma palmicola'

'*Candidatus* Phytoplasma palmicola' (palmi- *combining form* [L, fr. *palma* palm] -cola *n combining form* -s [NL, fr. L] **:** inhabitant, referring to the palm-inhabiting phytoplasma).

The reference strain LYDM-178^R is associated with succession of symptoms that include fruit abortion, inflorescence necrosis, foliar discoloration and mortality of coconut palm. [(*Mollicutes*) NC; NA; O, wall-less; NAS (GenBank accession number KF751386), oligonucleotide sequences

of unique regions of the 16S rRNA gene are: $_{169}$5'-AATGAGGCATCTCGTTAT-3'$_{186}$, $_{196}$5'-ACCTTCGCAAGAAGGT-3'$_{211}$, $_{448}$5'-CGCTAGTGGAAAAACTAGT-3'$_{466}$, and $_{1237}$5'-CAG CTGAAACGTGAGTTGTTAGCT-3'$_{1260}$; P (*Cocos nucifera*, phloem); M]. Harrison *et al*., this study.

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Table 1. '*Candidatus* Phytoplasma' species reference strains and their 16S rRNA gene RFLP group/subgroup classifications.

Strain name	GenBank accession no.	16S RFLP group classification*	16S rRNA gene sequence length (bp)	Sequence identity (%) with '*Ca*. Phytoplasma palmicola	Reference
Formally described '*Ca*. Phytoplasma' species					
'*Ca*. Phytoplasma palmicola' strain LYDM 178^{R}	KF751386	16SrXXII-A	1530	100	This study
'*Ca*. Phytoplasma asteris'	M30790	16Sr1-B	1530	89.3	Lee *et al.* (2004a)
'*Ca*. Phytoplasma aurantifolia'	U15442	16SrII-B	1513	90.7	Zreik *et al.* (1005)
'*Ca*. Phytoplasma australasia'	Y10097	16SrII-D	1521	90.8	White *et al.* (1998)
'*Ca*. Phytoplasma pruni'	JQ044393	16SrIII-A	1519	92.9	Davis *et al.* (2013)
'*Ca*. Phytoplasma ulmi'	AY197655	16SrV-B	1487	92.2	Lee *et al*. (2004b)
'*Ca*. Phytoplasma ziziphi'	AB052876	16SrV-B	1529	92.7	Jung *et al.* (2003b)
'*Ca*. Phytoplasma rubi'	AY197648	16SrV-D	1529	93.0	Malembic-Maher *et al.* (2011)
'*Ca*. Phytoplasma balanitae'	AB689678	ND	1528	92.9	Win *et al.* (2012)
'*Ca*. Phytoplasma trifolii'	AY390261	16SrVI-A	1531	93.4	Hiruki &Wang (2004)
'*Ca*. Phytoplasma sudamericanum'	GU292081	16SrVI-I	1530	93.1	Davis *et al.* (2012)
'*Ca*. Phytoplasma fraxini'	AF092209	16SrVII-A	1530	93.5	Griffiths *et al.* (1999)
'*Ca*. Phytoplasma phoenecium'	AF515636	16SrIX-D	1496	90.7	Verdin *et al.* (2003)
'*Ca*. Phytoplasma mali'	AJ542541	16SrX-A	1514	90.8	Seemüller & Schneider (2004)
'*Ca*. Phytoplasma pyri'	AJ542543	16SrX-C	1514	90.8	Seemüller & Schneider (2004)
'*Ca*. Phytoplasma spartii'	X92869	16SrX-D	1515	90.5	Marcone *et al.* (2004a)
'*Ca*. Phytoplasma prunorum'	AJ542544	16SrX-F	1514	90.7	Seemüller & Schneider (2004)
'*Ca*. Phytoplasma oryzae'	AB052873	16SrXI-A	1523	93.1	Jung *et al.* (2003)
'*Ca*. Phytoplasma solani'	AF248959	16SrXII-A	1524	88.4	Quaglino *et al.* (2013)
'*Ca*. Phytoplasma australiense'	L76865	16SrXII-B	1377	89.2	Davis *et al.* (1997)
'*Ca*. Phytoplasma japonicum'	AB010425	16SrXII-D	1521	89.6	Sawayanagi *et al.* (1999)
'*Ca*. Phytoplasma fragariae'	DQ086423	16SrXII-E	1333	88.6	Valiunas *et al.* (2006)

'Ca. Phytoplasma cynodontis'	AJ550984	16SrXIV-A	1501	93.1	Marcone *et al.* (2004b)
'Ca. Phytoplasma brasiliense'	AF147708	16SrXV-A	1528	90.2	Montano *et al.* 2001)
'Ca. Phytoplasma graminis'	AY725228	16SrXVI-A	1521	84.8	Arocha *et al.* (2005)
'Ca. Phytoplasma caricae'	AY725234	16SrXVII-A	1516	84.5	Arocha *et al.* (2005)
'Ca. Phytoplasma americanum'	DQ174122	16SrXVIII-A	1503	88.2	Lee *et al.* (2006)
'Ca. Phytoplasma castaneae'	AB054986	16SrXIX-A	1522	94.0	Jung *et al.* (2002)
'Ca. Phytoplasma rhamni'	X76431	16SrXX-A	1473	90.3	Marcone *et al.* (2004a)
'Ca. Phytoplasma pini'	AJ632155	16SrXXI-A	1528	93.4	Schneider *et al.* (2005)
'Ca. Phytoplasma omanense'	EF666051	16SrXXIX-A	1423	93.1	Al-Saady *et al*. (2008)
'Ca. Phytoplasma tamaricis'	FJ432664	16SrXXX-A	1516	90.6	Zhao *et al.* (2009a)
'Ca. Phytoplasma costaricanum'	HQ225630	16SrXXXI-A	1502	89.3	Lee *et al*. (2011)
'Ca. Phytoplasma malaysianum'	EU371934	16SrXXXII-A	1527	93.3	Nejat *et al.* (2013)
'Ca. Phytoplasma allocasuarinae'	AY135523	ND	1152	90.1	Marcone *et al.* (2004a)
'Ca. Phytoplasma lycopersici'	EF199549	ND	1523	87.1	Arocha *et al.* (2007)
'Ca. Phytoplasma convolvuli'	JN833705	ND	1496	89.1	Martini *et al.* (2012)
Provisional novel '*Ca*. Phytoplasma' species and other incidentally cited strains:					
'*Ca.* Phytoplasma palmae'	AF498307	16SrIV-A	1524	94.8	IRPCM (2004)
'Ca. Phytoplasma cocostanzaniae'	X80117	16SrIV-C	1524	94.9	IRPCM (2004)
Sabal palm decline phytoplasma	FJ217386	16SrIV-D	1441	94.7	Harrison *et al.* (2009)
'Ca. Phytoplasma vitis'	AF176319	16SrVIII-A	1529	93.2	IRPCM (2004)
'Ca. Phytoplasma luffae'	AF086621	16SrXII-A	1530	94.1	IRPCM (2004)
Strains of '*Ca*. Phytoplasma palmicola'					
LDN phytoplasma, Nigeria	Y14175	16SrXXII-A	1530	100	Tymon *et al.* (1998)
LYDM-182 phytoplasma, Mozambique	EU549768	16SrXXII-A	1530	100	This study
LYDM-185 phytoplasma, Mozambique	KF751388	16SrXXII-A	1530	100	This study
'*Ca*. Phytoplasma palmicola'-related strains:					

CSPW (LDG) phytoplasma,Ghana	Y13912	16SrXXII-B	1530	99.5	Tymon *et al.* (1998)
CSPW phytoplasma (DNA31), Côte d'Ivoire	KF387570	16SrXXII-B	1530	99.6	This study
CSPW phytoplasma (DNA19), Côte d'Ivoire	KF364359	16SrXXII-B	1530	99.6	This study
CSPW phytoplasma (DNA43), Côte d'Ivoire	KF419286	16SrXXII-B	1530	99.6	This study
CSPWB phytoplasma, Ghana	JQ868442	16SrXXII-B	1508	99.5	Makarova *et al.* (2012)
CILY phytoplasma, Côte d'Ivoire	KC999037	16SrXXII-B	1251	99.0	Konan Konan *et al.* (2013)

ND , Not determined

*16Sr RFLP group/subgroup classification status of the phytoplasma strains are based on Lee *et al*. (1998, 2000, 2006, 2011), Wei *et al*., (2007), Davis *et al*. (2013), Nejat *et al*. (2013).


CSPW phytoplasma, Côte d'Ivoire (KF419286)S
CSPW phytoplasma, Ghana (JQ868442)S
CSPW phytoplasma, Côte d'Ivoire (KF364359)S
CSPW phytoplasma, (CILY) Ivory Coast (KC999037)S
CSPWB phytoplasma, Côte d'Ivoire (KF387570)S
CSPW phytoplasma (LDG), Ghana (Y13912)S
'Ca. Phytoplasma palmicola' (KF751386)
'Coconut phytoplasma (M-182), Mozambique (EU549768)S
Awka wilt (LDN) phytoplasma, Nigeria (Y14175)S
Coconut phytoplasma (LYDM-185), Mozambique (KF751388)S
Coconut lethal disease phytoplasma, Tanzania (FJ217385)S
'Ca. Phytoplasma cocostanzaniae' (X80117)P
'Ca. Phytoplasma palmae' (AF498307)P
Sabal palmetto decline phytoplasma' (FJ217386)S
'Ca. Phytoplasma castaneae' (AB054986)
'Ca. Phytoplasma pini' (AJ632155)
'Ca. Phytoplasma cynodontis' (AJ550984)
'Ca. Phytoplasma oryzae' (D12581)
'Ca. Phytoplasma malaysianum' (EU371934)
'Ca. Phytoplasma luffae' (AF086621)P
'Ca. Phytoplasma sudamericanum' (GU292081)
'Ca. Phytoplasma fraxini' (AF092209)
'Ca. Phytoplasma trifolii' (AY390261)
'Ca. Phytoplasma ziziphi' (AY072722)
'Ca. Phytoplasma balanitae' (AB689678)
'Ca. Phytoplasma rubi' (AY197648)
'Ca. Phytoplasma vitis' (AF176319)P
'Ca. Phytoplasma ulmi' (AF122910)
'Ca. Phytoplasma omanense' (EF666051)
'Ca. Phytoplasma phoenecium' (AF515636)
'Ca. Phytoplasma pruni' (JQ044393)
'Ca. Phytoplasma brasiliense' (AF147708)
'Ca. Phytoplasma aurantifolia' (U15442)
'Ca. Phytoplasma australasia' (Y10097)
'Ca. Phytoplasma costaricanum' (HQ225630)
'Ca. Phytoplasma asteris' (M30790)
'Ca. Phytoplasma lycopersici' (EF199549)
'Ca. Phytoplasma fragariae' (DQ086423)
'Ca. Phytoplasma japonicum' (AB010425)
'Ca. Phytoplasma australiense' (L76865)
'Ca. Phytoplasma americanum' (DQ174122)
'Ca. Phytoplasma convolvuli' J(N833705)
'Ca. Phytoplasma solani '(AJ964960)
'Ca. Phytoplasma caricae' (AY725234)
'Ca. Phytoplasma graminis' (AY725228)
'Ca. Phytoplasma rhamni' (X76431)
'Ca. Phytoplasma tamaricis' (FJ432664)
'Ca. Phytoplasma spartii' (X92869)
'Ca. Phytoplasma prunorum' (AJ542544)
'Ca. Phytoplasma mali' (AJ542541)
'Ca. Phytoplasma pyri' (AJ542543)
Acholeplasma palmae (L33734)
20

Fig. 1. Phylogenetic tree inferred from analysis of 16S rRNA gene sequences. Maximum-parsimony analysis was performed using the close neighbour interchange (CNI) algorithm in MEGA4. The initial tree for the CNI search was obtained with the random addition of sequences (10 replicates). The reliability of the analysis was subjected to a bootstrap test with 1000 replicates. The percentages (>60) of replicate trees in which the associated taxa clustered together in the bootstrap analysis are shown next to the branches. The taxa used in the phylogenetic tree construction included '*Ca*. Phytoplasma palmicola' reference strain (underlined), reference strains of 36 previously described '*Ca*. Phytoplasma' species, provisional '*Ca*. Phytoplasma' species (labelled with superscript **P**), and other incidental strains (labeled with superscript **S**). *Acholeplasma palmae* served as an outgroup during the phylogenetic tree construction. Bar, 20 nt substitutions.

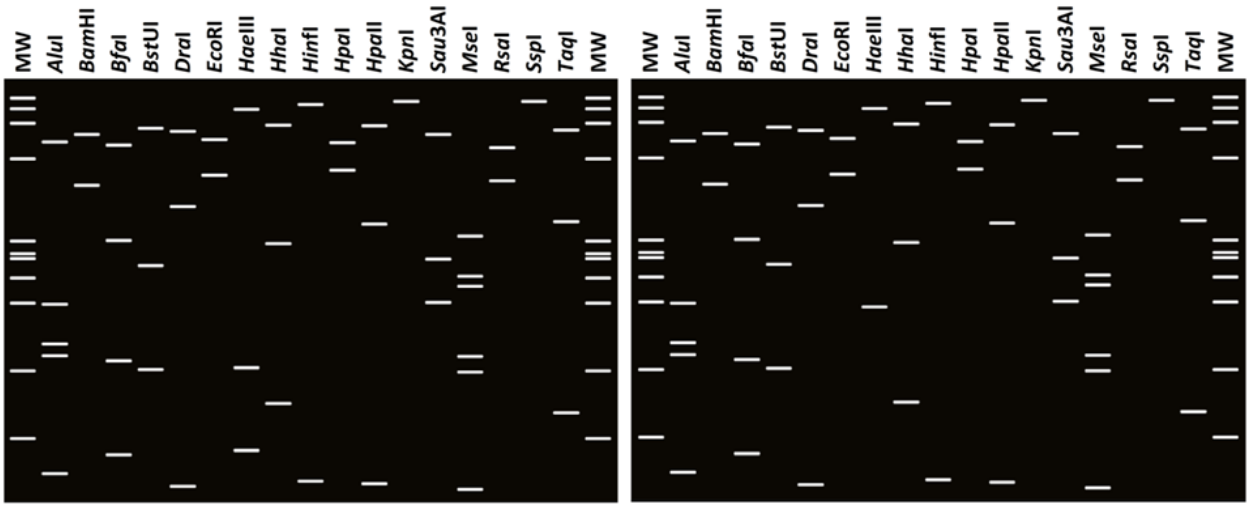

Fig. 2. Virtual RFLP patterns derived from *in silico* digestions, using pDRAW32 of F2n/R2 fragments phytoplasma the 16S rRNA gene with 17 restriction endonuclease enzymes: *Alu*I, *Bam*HI, *Bfa*I, *Bst*UI, *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hin*fI, *Hpa*I, *Hpa*II, *Kpn*I, *Sau*3AI, *Mse*I, *Rsa*I, *Ssp*I, and *Taq*I. The restriction fragments were resolved by *in silico* electrophoresis through a 3% agarose gel. Subgroup 16SrXXII-A coconut phytoplasma strain LYDM-178^{R} (KF751386) (left) and subgroup 16SrXXII-B phytoplasma strain CSPWB-DNA43 (KF419286) (right). MW, ΦX174 DNA–HaeIII digest.